Exercise 10
Rh Immune Globulin Workup (RhIgW)

1. State the purpose for giving Rh Immune Globulin (RhIg).
2. State the population which is most frequently given RhIg.
3. State the severity of HDFN.
4. State the bilirubin level which is considered critical.
5. Define “kernicterus”.
6. Describe how RhIg presents sensitization to the D antigen.
7. State the amount of anti-D present and the volume of D positive RBCs (both packed and whole blood) that this will cover and within what time period this must be administered.
8. State when the doses of RhIg are administered.
9. State 3 populations of women who are not RhIg candidates.
10. List 7 circumstances when a pregnant D negative patient must be given RhIg.
11. State the course of action which must be taken, so far as RhIg administration, when the D type of an infant cannot be determined.
12. State the testing which must be performed on the mother of a D positive infant to determine RhIg candidacy and the results of each.
13. State the appearance of the weak D test which may indicate that an excessive feto-maternal hemorrhage has occurred.
14. State the immune antibody, which if present in the mother, would cause her to be ineligible for RhIg.
15. State the principle of the erythrocyte rosette test (fetal bleed screen) including the appearance of positive and negative results.
16. State the next step when a positive fetal bleed screen is resulted.
17. State the principle of the Kleihauer-Betke acid elution stain including: controls which must be run and the appearance of positive and negative results.
18. Given the results of a Kleihauer-Betke acid elution test correctly calculate the number of vials of RhIg which must be administered.
19. State the D type of an infant which would require a Kleihauer-Betke test to be performed instead of the fetal bleed screen.
20. Accurately perform cord blood DATs and additional testing as necessary to determine if a RhIg workup must be performed.
21. Correctly perform a RhIg workup on a maternal sample.
22. Based on the results of the RhIg workup state the number of vials of RhIg which must be administered.
23. Given the results of RhIg work up state additional testing which must be performed.
24. Given the results of RhIg work ups determine whether or not the patient is a candidate for RhIg.

Introduction
Rh immune globulin (RhIg) is a product which contains high-titered IgG D antibodies of human origin which is commercially available for use in preventing alloimmunization of D negative individuals after exposure to the D antigen.
One group at increased risk of exposure and sensitization by the D antigen are D negative mothers who give birth to D positive infants. During delivery of the baby, Rh positive fetal cells enter the maternal circulation when the placenta separates from the uterine wall. In general, only a small amount of fetal blood (less than 30 ml whole blood) enters the maternal circulation, but due to the immunogenicity of the D antigen, this is enough to cause sensitization. If a sensitized woman becomes pregnant with another D positive baby, the baby will be affected with severe HDFN.

HDFN due to anti-D is the most severe type. The bilirubin level rises quickly after birth. If it exceeds 20 mg/dL kernicterus may occur. Kernicterus is caused when the basal ganglia and other areas of the brain and spinal cord are infiltrated with bilirubin resulting in mental retardation or death if not treated. In addition to the high bilirubin levels, these infants become severely anemic due to the destruction of their red blood cells by the maternal antibody.

To prevent sensitization to the D antigen, Rh immune globulin (Rhlg) was developed and became available in the early 1960s. This product contains anti-D which, when injected intramuscularly within 72 hours following delivery of an Rh positive baby, will attach to the D antigen on the baby's RBCs. This would result in the baby's cells being recognized as foreign and removed from the mother's circulation before her immune system could recognize the D antigen as foreign, thus preventing her from becoming immunized. One vial of Rhlg contains 300 ug of anti-D, enough to cover a 30 ml whole blood bleed or 15 mLs of packed cells.

Later, it was discovered that even though the D negative mothers were identified and given Rhlg within the appropriate time frame, there was still a 1% failure rate. That is to say, one per cent of the mothers who received Rhlg after delivery were subsequently discovered to be immunized to the D antigen. It was then determined that very small fetal-maternal hemorrhages were occurring during the third trimester. It is now routine practice for physicians to administer a dose of Rhlg at 28 weeks gestation, in addition to the postpartum dose. Antepartum administration of Rhlg has decreased the overall risk of immunization to 0.1%. No adverse effects have been observed in infants of mothers who have received up to two doses of antenatal Rhlg although it may cause the baby to have a positive DAT.

After delivery of an infant, certain tests are performed to determine whether or not a woman is to be considered a Rhlg candidate. The following women are not candidates for Rhlg:

1. D negative women who have D negative babies
2. D positive women
3. D negative women known to be immunized to D.

Rhlg must be given to D negative women under the following circumstances in which the baby's D is unknown:

1. Post miscarriage/abortion
2. Post ectopic pregnancy
3. Vaginal bleeding at any time during the pregnancy
4. Fetal death
5. Post amniocentesis
6. Chorionic villus sampling
7. Cordocentesis
8. After an external cephalic version of a breech fetus
9. Blunt trauma to the abdomen
RhIg must also be given if the D type of the infant cannot be determined after birth as may be due to the infant having a positive DAT. In these situations the D and/or Rh control tubes are positive at the AHG phase during weak testing. This is the reason that a D control or DAT must be performed on any individual who appears weak D positive. Unless the mother is D positive, the best rule of thumb to follow is when in doubt, give it.

After a D negative woman gives birth to an infant, an ABO/D type is ordered on the infant's cord blood. A DAT may also be ordered. If the baby is D positive, an Rh immune globulin (RhIg) work up is ordered on the mother. The RhIg work up includes:

1. ABO/D typing, including weak D - may be performed on sample collected on admission or post-partum sample.
2. Antibody screen - may be performed on sample collected on admission or post-partum sample.
3. Fetal Bleed Screen/Rosette procedure - performed on post-partum sample

Some facilities perform the type and screen upon admission. This test would NOT be repeated on the post-partum sample. For today’s activities you will perform each. Each test serves a specific purpose:

1. **ABO/D type** - It is very important to accurately determine the D type of the mother. If a mother is D positive or weak D positive, she is not a RhIg candidate. The weak D must be read microscopically if required by the facility. This is one way in which an excessive feto-maternal hemorrhage (FMH) may be detected. If excessive D positive fetal cells are in the maternal circulation they will be coated by the anti-D and form clumps when the AHG serum is added. A “mixed-field” appearance in the anti-D tube and a negative Rh control is the first indication that an excessive bleed has occurred.

2. **Antibody screen** - An antibody screen is routinely performed to identify those women who have formed or are forming an immune anti-D. Occasionally a mother's screens may be positive due to the antepartum administration of the RhIg. In these cases the antibody screen is very, very weak and once identified as being due to RhIg administration; she must still get a postpartum dose. If there is doubt as to whether the anti-D present is immune or passively acquired due to antepartum administration of RhIg a titer should be performed. *Passively acquired anti-D rarely achieves a titer above 4.* If it is determined that the anti-D is immune, she is not a RhIg candidate. It is critical that her history be obtained, specifically when her dose of antepartum RhIg was administered. The term used for the anti-D detected after administration of RhIg is “passively acquired anti-D”.

3. **Erythrocyte Rosette Test** – The brand name of the kit used in lab is “FMH Rapid Screen”. This test is performed to screen for an abnormal large feto-maternal hemorrhage which would indicate the need for more than 1 vial of RhIg. If the test is positive it indicates an excessive feto-maternal hemorrhage may have occurred and one vial of RhIg may be insufficient. A Kleihauer-Betke acid elution stain must be performed to determine the amount of the bleed. Once the amount is determined a calculation is performed to determine the number of vials needed.
**Principle of the Rosette Test**
The rosette test is a sensitive method to detect FMH of approximately 10 mL or more. A suspension of red cells from a *post-partum* blood sample collected from the D negative mother is mixed with antibody reagent (anti-D), incubated, and washed to remove unbound antibody. During the incubation period, any D positive fetal red cells present will become sensitized with anti-D. One antigen binding site of each antibody molecule (Fab piece) attaches to a fetal cell leaving the other antibody site free to attach to a D antigen of the Fetal Bleed Screening Indicator Cells (D positive cells) which are subsequently added. The suspension of test cells and indicator cells is centrifuged briefly, transferred to a microscope slide, or read the tube on the tube holder and examined for clumps of agglutinated red cells, using low power magnification.

The result is an appearance of rosettes or mixed-field agglutinates in which the agglutinates consist of clumps of D positive indicator cells clustered about the sensitized, D positive fetal cells. Each clump seen in the rosette test represents **ONE fetal cell** surrounded by the indicator cells. If a clump of cells is seen in the **weak D test, each cell in the clump is a fetal cell**. The rosette test is so sensitive and specific that there must be a certain number of clumps seen before it is called positive, while in the weak D the presence of even one clump is significant. When the test sample contains few or no D positive fetal cells, rosettes may not be observed. Because the vast majority of samples screened will contain very few D positive fetal red cells, observing measurable numbers of rosettes in a test should alert the technologist to the possibility that a larger than expected fetal-maternal hemorrhage may have occurred. In this situation, the Kleihauer-Betke must be used to determine the amount of fetal-maternal hemorrhage and the dosage of Rh immune globulin required.

A positive rosette test indicates the occurrence of a large FMH. While estimates suggest that only 0.3% of pregnancies are complicated by FMH greater than 30 mL, a large FMH is an important and preventable cause of failed immunoprophylaxis.

If the newborn types as weak D positive the red cells may not react as well as D positive red cells in the rosette procedure, presumably because they have fewer D antigen sites. **If the newborn types as Rh weak D positive, a Kleihauer-Betke acid elution test should be done routinely.**

The Rosette procedure is much more sensitive than the weak D in detecting excessive fetal-maternal hemorrhages.

A positive weak D and/or rosette procedure indicates that an excessive fetal-maternal hemorrhage has occurred. The weak D and rosette are *qualitative* tests *screening* tests. They can determine that an excessive bleed has occurred, but not how much.

If an excessive feto-maternal hemorrhage is indicated by a positive rosette test a quantitative test such as the Kleihauer-Betke acid elution test or flow cytometry must be performed to accurately calculate the dos of RhIg.
EXERCISE 10

**Principle of the Kleihauer-Betke Acid Elution Test**
The most common method used to *quantitate* the bleed is the **Kleihauer-Betke acid elution stain**. The principle is based on the fact that fetal hemoglobin (hemoglobin F) is resistant to acid elution, whereas adult hemoglobin is not. When a thin blood smear is exposed to an acid buffer, the adult red blood cells lose their hemoglobin into the buffer so that only stroma remains. Normal adult cells appear as very pale ghosts. Fetal red cells are unaffected and retain their hemoglobin. Fetal cells appear as bright pink refractile bodies.

The volume of fetal cells is determined by recording the number of fetal cells observed in 2000 adult cells and converting it to percentage using either of the following formulas:

1. **Number of fetal cells** x 100 = percent fetal cells
   2,000

   \[
   \text{% fetal cells} \times 50 = \text{volume of feto-maternal hemorrhage}
   \]

2. **Fetal cells/2000 x 5000mL = fetal whole blood**
   **One vial of RhIg will cover a 30 ml whole blood bleed.** If it is determined that the bleed exceeds this amount, the volume of fetal bleed is divided by 30 to determine the number of doses of RhIg required.

   \[
   \frac{\text{Fetal whole blood}}{30} = \text{number of vials}
   \]

Because of the inherent error of the Kleihauer-Betke test if the calculated dose to the right of the decimal point is $\geq 0.5$ vial, round up to the next whole number and add a vial. If $<0.5$, round down and add one vial.

**EXAMPLE:**

Six fetal cells were counted in 2000 adult cells in a Kleihauer-Betke acid elution test:

6 cells/2000 cells x 5000mL = 15 mL of fetal whole blood.

15/30 = 0.5 vial

Round up to 1 and add 1 = 2 vials to be given.
Closing Comments
Many sites will perform the Type and Screen on the maternal sample upon admission. The determination of the D type of the cord blood may be done on one shift, the D negative women needing RhIg identified, and the maternal post-partum sample for the fetal bleed screening test will be drawn with her morning CBC. The fetal bleed screen test is performed on the post-partum sample, while the T/S is usually while before delivery.

In this lab you will do all testing, cord blood, type and screen on mom and fetal bleed screen test. But remember, in the “real” world each of these tests may be done by other individuals on different shifts.
Specimen
The test procedure requires a blood specimen collected from the MOTHER after delivery of all products of conception. It is best to wait at least one hour after delivery to allow any fetal blood to mix thoroughly in the maternal circulation. Blood is drawn into an anticoagulant such as EDTA. Store at 1-8°C for no longer than two days. The test should be carried out as quickly as possible to permit administration of RhIG within 72 hours.

Procedure

Cord Blood
1. Perform DAT on cord bloods as in DAT exercise. Record GRADED reactions.
2. Perform ABO/D typing on the cord bloods of all D negative mothers.
3. If mother is D negative and baby is D positive, obtain maternal specimen from instructor for RhIG work up.

Maternal Blood - Take care and DO NOT use the CORD BLOOD which comes from BABY.
1. Place maternal serum in appropriately labeled tube or, if provided, label the tube provided and prepare a 4-6% patient cell suspension (mother’s cells). IMPORTANT: Use the MATERNAL sample for the work up and take care to not over-dilute your cell suspension!!
2. Perform a type and screen on the mother. Remember to set up D ctrl tube.

While the type and screen is incubating, perform the FMH RapidScreen.
1. Use the cell suspension prepared to the type and screen on the mother.
2. Place 1 drop of the prepared maternal red blood cell suspension in a properly labeled test tube.
3. Mix well and incubate for 5 minutes (+/- 1 minute) at room temperature (18°C to 30°C).
4. Wash the red blood cells four times with saline, being careful to decant the saline completely between washes and to resuspend the red blood cells thoroughly when adding saline for the next wash.
5. Decant the saline completely after the last wash.
6. Add one drop of Indicator Cells and mix well by gently shaking the tube.
7. Centrifuge immediately for the 15 seconds at 3400rpm or a time appropriate to the calibration of the centrifuge.
8. Resuspend the red blood cell button completely and examine five (5) low-power fields microscopically for mixed-field agglutination using 100x magnification. Read the controls first.
   a. After examination of five low-power fields, if five or more rosettes of red blood cells are observed, the test is positive and indicates the presence of D-positive fetal red blood cells in possibly significant numbers in the maternal blood.
   b. After examination of five low-power fields, if four or fewer rosettes of red blood cells are observed, the test is negative, indicating that a large feto-maternal hemorrhage did not occur.
9. Record your reactions in the FBS portion of the recording results form. Since this is a QUALITATIVE test the patient and controls are recorded as either “positive” or “negative”.
Interpretation of Results

- **Antibody screen** – Refer to previous procedure.

- **Weak D Test**

<table>
<thead>
<tr>
<th>Anti-D (IS)</th>
<th>Weak D</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>O</td>
<td><strong>D negative</strong></td>
</tr>
<tr>
<td>O</td>
<td>3+</td>
<td><strong>Weak D positive - not a RhIg candidate</strong></td>
</tr>
<tr>
<td>O</td>
<td>±MF</td>
<td><strong>Possible excessive feto-maternal hemorrhage – perform Kleihauer-Betke test</strong></td>
</tr>
</tbody>
</table>

- **Fetal Bleed Screen Test**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Positive Control</th>
<th>Negative Control</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Four or fewer rosettes</td>
<td>+</td>
<td>O</td>
<td><strong>Negative - excessive bleed has not occurred</strong></td>
</tr>
<tr>
<td>in five fields</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Five or more rosettes</td>
<td>+</td>
<td>O</td>
<td><strong>Positive - excessive bleed has occurred, perform Kleihauer-Betke test</strong></td>
</tr>
<tr>
<td>in five fields</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>+</td>
<td>+</td>
<td>Invalid, repeat test</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Invalid, repeat test</td>
</tr>
<tr>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Invalid, repeat test</td>
</tr>
</tbody>
</table>
**Cord Blood Flow Chart**

**Direct Antiglobulin Test – DAT**

<table>
<thead>
<tr>
<th>DAT Negative</th>
<th>DAT Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mom D pos</td>
<td>ABO/D type cord cells</td>
</tr>
<tr>
<td>Mom D Neg</td>
<td>Type and Screen Mom</td>
</tr>
<tr>
<td>Nothing more</td>
<td>Screen Negative and Mom is Group O and baby is A or B</td>
</tr>
<tr>
<td></td>
<td>Screen Positive</td>
</tr>
<tr>
<td>D neg baby</td>
<td>Panel on mom Identify antibody in mom's serum</td>
</tr>
<tr>
<td>No RhIg</td>
<td>Acid eluate on cord cells</td>
</tr>
<tr>
<td>RhIg needed for Mom</td>
<td>Identify antibody in cord eluate</td>
</tr>
</tbody>
</table>

For D negative mothers in whom the baby has a positive DAT, RhIg candidy is determined by identification of the antibody coating the baby's cells.

- If it is due to *immune* anti-D (determined by mother's history) she is *not* a RhIg candidate.
- If it is due to passively acquired anti-D from her antenatal dose of RhIg (determined by mother's history) she *is* a RhIg candidate.
- If it is due to any other immune antibody (i.e., K, Fy, A etc.) she *is* a RhIg candidate.
**Exercise 10**

**Cord Blood Recording Results**

**IMPORTANT NOTE:**
1. Only perform ABO/D typing on cord cells **IF INDICATED**.
2. Only answer RhIg question IF MOM IS D NEGATIVE.
3. Points will be deducted for unnecessary testing or answers.

<table>
<thead>
<tr>
<th>Mom’s Name</th>
<th>MR#</th>
<th>Mom’s ABO/D</th>
<th>RhIg Ye or No</th>
<th>Cord DAT</th>
<th>✅ Cells</th>
<th>Cord Cell Typing with Anti-</th>
<th>ABO/D</th>
<th>Tech</th>
<th>Date</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>DC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. 
2. 
3. 
4.
Exercise 10
Rh Immune Globulin (RhIG) Work
Study Questions

1. Describe how a D-negative mother is exposed to the D antigen during the birth process. (1 point)

2. Define “kernicterus”. (1 point)

3. State the quantity of anti-D in one vial of Rh immune globulin (RhIG). (1 point)

4. State how an administration of RhIG prevents sensitization of the mother to the D antigen and within what time frame must it be administered. (2 points)

5. State the whole blood and packed cell volumes that 1 vial of RhIG will cover. (1 point)

6. State the reason for administration of ante-partum administration of RhIG and when during the pregnancy this is administered. (1 point)

7. List three (3) groups of women who are NOT candidates for RhIG? (1.5 points)

8. List seven (7) circumstances when an Rh negative woman should receive RhIG? (3.5 points)
   a.
   b.
   c.
   d.
   e.
   f.
   g.

9. What group of tests constitutes a RhIG work up AND briefly state the purpose of each test performed. (3 points)
10. What is the term used for anti-D present in the maternal serum due to administration of RhIG? What is the term used for an anti-D present in a D negative person who has been sensitized to the D antigen? (1 point)  
a.  
b.

11. Briefly describe the principle of the Rosette test? (2 points)

12. Briefly describe the principle of the Kleihauer-Betke acid elution test? (2 points)

13. State why the rosette test more sensitive and specific than the weak D test. (1 point)

14. A Kleihauer-Betke acid elution test was done. The results are 50 fetal cells in 2000 adult cells seen. Calculate the dose of RhIG needed. Show your work. (1 point)

15. How is it determined whether the anti-D detected in the maternal sample is immune or passively acquired? (1 point)

16. For each of the following determine whether or not the Mother is a RhIG candidate. (3 Points)

<table>
<thead>
<tr>
<th>Mmaternal ABO/D</th>
<th>Mmaternal Antibody Screen</th>
<th>Infant ABO/D</th>
<th>DAT</th>
<th>RhIG Yes or No</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Negative</td>
<td>Negative</td>
<td>0 Negative</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>A Positive</td>
<td>Negative</td>
<td>0 Negative</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>A Negative</td>
<td>Anti-C and Anti-K</td>
<td>A Positive</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>0 Negative</td>
<td>Negative</td>
<td>B Positive</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>A Negative</td>
<td>Passively acquired anti -D</td>
<td>0 Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>A B Negative</td>
<td>Anti-D due to sensitization</td>
<td>A Positive</td>
<td>Positive</td>
<td></td>
</tr>
</tbody>
</table>